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# Synthesis of Cytochrome c Oxidase 2: a p53-dependent metabolic regulator that promotes respiratory function and protects glioma and colon cancer cells from hypoxia-induced cell death

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**Running Title:** SCO2 inhibits hypoxia-induced cell death

**Key words:** p53, SCO2, hypoxia, glioma, respiration

**Abbreviations:**

AMPKα, AMP-activated protein kinase α; ACC, actetyl coA carboxylase; COX, cytochrome c oxidase; EGFR, epidermal growth factor receptor; 4-EBP1, 4E binding protein 1; GLUT1, glucose transporter 1; hygro, hygromycin; luc, luciferase; p53ts, temperature-sensitive p53; PI, propidium iodide; PI3K, phosphatidylinositol-3 kinase; PKB/Akt, protein kinase B; phospho, phosphorylated; puro, puromycin; ROS, reactive oxygen species; SCO2, Synthesis

of Cytochrome c Oxidase 2; shRNA, short hairpin RNA; siRNA, small interfering RNA; TIGAR, tp53 inducer and regulator of glycolysis, wt, wildtype.

**Abstract**

p53 has an important role in the processing of starvation signals. p53-dependent molecular mediators of the Warburg effect reduce glucose consumption and promote mitochondrial function. We therefore hypothesized that the retention of wild-type p53 characteristic of primary glioblastomas limits metabolic demands induced by deregulated signal transduction in the presence of hypoxia and nutrient depletion. Here we report that shRNA-mediated gene suppression of wild-type p53 or ectopic expression of mutant temperature-sensitive dominant-negative $p53^{V135A}$ increased glucose consumption and lactate production, decreased oxygen consumption and enhanced hypoxia-induced cell death in p53 wild-type human glioblastoma cells. Similarly, genetic knock-out of p53 in HCT116 colon carcinoma cells resulted in reduced respiration and hypersensitivity towards hypoxia-induced cell death. Further, wild-type p53 gene silencing reduced the expression of *Synthesis of Cytochrome c Oxidase 2* (SCO2), an effector necessary for respiratory chain function. A SCO2 transgene reverted the metabolic phenotype and restored resistance towards hypoxia in p53-depleted and in p53 mutant glioma cells in a rotenone-sensitive fashion, demonstrating that this effect was dependent on intact oxidative phosphorylation. Supplementation with methyl-pyruvate, a mitochondrial substrate, rescued p53 wild-type but not p53 mutant cells from hypoxic cell death, demonstrating a p53-mediated selective aptitude to metabolize mitochondrial substrates. Further, SCO2 gene silencing in p53 wild-type glioma cells sensitized these cells towards hypoxia. Finally, lentiviral gene suppression of SCO2 significantly enhanced tumor necrosis in a subcutaneous HCT 116 xenograft tumor model, compatible with impaired energy metabolism in these cells. These findings demonstrate that glioma and colon cancer cells with p53 wild-type status can skew the Warburg effect and thereby reduce their

vulnerability towards tumor hypoxia in a SCO2-dependent fashion. Targeting SCO2 may therefore represent a valuable strategy to enhance sensitivity towards hypoxia and may complement strategies targeting glucose metabolism.

## Introduction

Alteration of metabolism in tumor cells is increasingly recognized as a core component of the neoplastic phenotype that complements the classical hallmarks of cancer (Gottlieb, 2009; Hanahan and Weinberg, 2000). Otto Warburg in the early 1920s defined the phenomenon of aerobic glycolysis (Warburg, 1956a; Warburg, 1956b) characteristic of cancer cells preferentially utilizing the glycolytic pathway in the presence of oxygen, although less ATP per mole glucose is produced by glycolysis than by mitochondrial respiration. It has become evident, however, that increased glycolysis can be advantageous for tumors cells in many ways (DeBerardinis *et al.*, 2008; Gatenby and Gillies, 2004; Kondoh *et al.*, 2005; Moreno-Sanchez *et al.*, 2007). First, as long as sufficient glucose is available, energy demand can be satisfied by enhanced glycolytic activity, because the glycolytic flux involves fewer biochemical reactions than cellular respiration (Curi *et al.*, 1988; Gatenby and Gillies, 2004; Guppy *et al.*, 1993). Additionally, glycolysis provides cells with intermediate products needed for biosynthetic pathways including riboses for nucleotide setup and glycerate or citrate for lipid synthesis. Furthermore, the pentose phosphate pathway generates NADPH, acting as energy source and reducing agent for protection against reactive oxygen species (ROS). Several aspects of the molecular mechanisms underlying the glycolytic phenotype have recently been elucidated. Signaling downstream of receptor tyrosine kinases influences glucose metabolism. In particular, the phosphatidylinositol-3 kinase (PI3K)/protein kinase B (PKB)/Akt pathway that is activated in the vast majority of primary glioblastomas (Choe *et al.*, 2003) increases glycolytic activity by upregulation of glucose transporter 1 (GLUT1) (Barnes *et al.*, 2002) and phosphorylation of hexokinase-II (Miyamoto *et al.*, 2008).

Another event important for the development of cancer is the inactivation of the tumor suppressor p53 (Vogelstein *et al.*, 2000). Depending on the cellular conditions, p53 suppresses tumorigenesis by multiple mechanisms, including cell cycle regulation, initiation of DNA repair, induction of apoptosis, activation of autophagy or protection from autophagy-induced cell death and regulation of ROS production. Recently, however, p53 has been demonstrated to act also as a potent metabolic regulator. The p53-dependent metabolic response is driven by the starvation-sensitive AMP-activated protein kinase (AMPK) pathway (Jones *et al.*, 2005). At least two effectors downstream of p53 have been identified. First, p53 maintains mitochondrial respiration by inducing *Synthesis of Cytochrome c Oxidase 2* (SCO2) (Matoba *et al.*, 2006). SCO2 is essential for the proper assembly of cytochrome c oxidase (COX) which catalyzes the transfer of reducing equivalents from cytochrome c to molecular oxygen in the inner mitochondrial membrane. Loss of p53 or SCO2 results in a shift of metabolism towards glycolysis (Corcoran *et al.*, 2006; Ma *et al.*, 2007; Matoba *et al.*, 2006). Additionally, p53 may suppress glycolysis and increase the activity of the pentose phosphate shunt via induction of *tp53 inducer and regulator of glycolysis* (TIGAR) (Bensaad *et al.*, 2006).

Glioblastomas are amongst the most hypoxic human tumors, and primary glioblastomas, in particular, feature extensive necrosis. It is intriguing that almost all these tumors retain wild-type p53 status in contrast to secondary glioblastomas arising from low-grade gliomas, in which mutation of p53 is frequent (Tohma *et al.*, 1998; Watanabe *et al.*, 1996). We therefore hypothesized that wild-type p53 in glioma cells limits metabolic demands induced by deregulated signal transduction processes in the presence of hypoxia and nutrient depletion and thereby enhances viability under the adverse conditions of the glioblastoma microenvironment.

We here report that antagonism of p53 enhances the glycolytic phenotype and promotes hypoxia-induced cell death in a SCO2 dependent manner. SCO2 gene silencing mimicks the

effects of p53 suppression *in vitro*, while overexpression of SCO2 in cells with inactivated p53 confers the phenotype of p53 wild-type cells.

## Results

### Antagonizing p53 enhances hypoxia-induced cell death.

To analyze the importance of p53 for the ability of glioma cells to survive hypoxic stress, three models of p53 inactivation were used. (i) LNT-229 glioma cells stably transfected with a short-hairpin RNA targeting p53 (p53sh), (ii) LNT-229 cells in which the p53 transactivation activity was inhibited by stable expression of the temperature-sensitive dominant-negative $p53^{V135A}$ mutant (Naumann *et al.*, 1998) and iii) HCT116 human colon carcinoma cells carrying a targeted deletion of p53 achieved by homologous recombination ($p53^{-/-}$) and appropriate control cells (Bunz *et al.*, 1998; Ryan *et al.*, 1993). In a first step, we analyzed the expression of p53 in tumor cells exposed to limited glucose availability (2 mM) and different oxygen concentrations (21%, 1% or 0.1% $O_2$) (Fig. 1A). Hypoxia had no consistent effect on p53 protein levels. p53 protein levels in LNT-229 cells stably transfected with the p53sh sequence (LNT-229 p53sh cells) were efficiently reduced under all conditions. In LNT-229 p53ts cells, the murine transgenic p53 was detectable in addition to the endogenous human p53. Again, hypoxia had no effect on endogenous (p53sc263-antibody) or on transgenic (p53Ab4-antibody) p53 protein levels. No p53 was detectable in HCT116 $p53^{-/-}$ cells. Since conflicting results concerning transcriptional activity of p53 under hypoxia exist (Liu *et al.*, 2007; Zhao *et al.*, 2009), p53 transactivation activity under the experimental conditions was analyzed by luciferase assay. The p53-transactivation capacity was significantly reduced in p53-depleted cells (LNT-229 p53sh, LNT-229 p53ts and HCT116 $p53^{-/-}$) at all experimental conditions (Fig. 1B). Furthermore, hypoxia *per se* did not significantly alter the p53 transactivation activity. These results demonstrate that the expression of p53 and its transactivation activity are retained under starving conditions of hypoxia and glucose

deprivation. To test whether suppression of p53 modulates the sensitivity towards hypoxia-induced cell death, LNT-229 puro/p53sh, LNT-229 hygro/p53ts and HCT116 $p53^{+/+}$/$p53^{-/-}$ cells were exposed to glucose restriction (2 mM) and different oxygen concentrations (21%, 1% or 0.1% $O_2$) for 24 h, and cell death was assessed by propidium iodide (PI) staining. In all three paradigms the inactivation of p53 sensitized the cells towards hypoxia-induced cell death (Fig. 1C), an effect that was most prominent at moderate hypoxia.

**p53 is a regulator of glucose consumption in tumor cells.**

Since p53 plays a regulatory role in glucose metabolism during stress situations (Bensaad *et al.*, 2006; Corcoran *et al.*, 2006; Kondoh *et al.*, 2005; Matoba *et al.*, 2006), we hypothesized that suppression of wild-type p53 resulted in a higher nutrient demand under starvation conditions. This would accelerate nutrient depletion and promote earlier cell death. We compared glucose consumption and lactate production in LNT-229 puro/p53sh or HCT $p53^{+/+}$/$p53^{-/-}$ cells in normoxia (21% $O_2$) and hypoxia ((1% or 0,1% $O_2$). p53-depleted cells showed an increased glucose consumption and lactate production under all conditions tested independent of oxygen supply (Fig. 2 A-D). Similar results were obtained with LNT-229 hygro/p53ts cells (data not shown). Therefore, p53 deletion leads to an increased glucose consumption and consequently to an accelerated glucose depletion.

**Inhibiting p53 function represses cellular respiration.**

Recent work has elucidated that p53 enhances cellular respiration through transcriptional upregulation of SCO2, which is essential for the assembly of COX and thus maintains oxidative phosphorylation (Matoba *et al.*, 2006). As p53-antagonized cells were more sensitive towards hypoxia, we hypothesized that this was elicited by regulation of SCO2. We therefore determined SCO2 expression by qRT-PCR analysis in these cell lines. Antagonizing p53 repressed SCO2 in glioma and colon carcinoma cells (Fig. 3A). To assess the functional

consequences of p53 deletion on cellular respiration, oxygen consumption was directly measured in p53-suppressed and control cell lines. As shown in Fig. 3B, loss of p53 wild-type activity led to a considerable reduction in oxygen consumption, confirming a regulatory function of p53 for cellular respiratory activity.

**SCO2 is a p53 effector that maintains respiration and reduces sensitivity towards hypoxic stress**

To characterize the role of SCO2 as an effector of p53, an SCO2 expression vector was transfected into LNT-229, LNT-229 p53sh or T98G (p53 mutant) cells. Additionally, expression of SCO2 was transiently suppressed by siRNA in parental LNT-229 cells (Fig. 4A). Overexpression of SCO2 enhanced oxygen consumption in parental cells and rescued the suppression of respiration observed in p53sh cells, while gene suppression of SCO2 repressed respiration (Fig. 4B). To test whether restoration of SCO2 would also reconstitute resistance of p53-depleted cells towards hypoxia-induced cell death, p53sh cells transfected with SCO2 or control vector were incubated at 21%, 1% or 0.1% $O_2$ and cell death was assessed thereafter. Under moderately hypoxic conditions (1% $O_2$), ectopic expression of SCO2 protected wild-type LNT-229 cells from hypoxia-induced cell death and reverted the hypersensitivity of p53-depleted cells (Fig. 4C). Under profoundly hypoxic conditions (0.1% $O_2$), however, the SCO2 transgene did not convey protection from hypoxia. Similarly, gene suppression of SCO2 enhanced the sensitivity of LNT-229 cells towards cell death only at moderate hypoxia, indicating that concentrations of oxygen sufficient for electron transfer within the mitochondrial respiratory chain are a prerequisite for the hypoxia-protective effects of SCO2 (Fig. 4C). Similar results were obtained with LN-18 (mutant p53) and LNT-229 hygro/p53ts cells (data not shown).

**A functional respiratory chain is required for the SCO2-mediated protection**

To determine whether the effect of SCO2 on hypoxia-induced cell death depends on its function related to the mitochondrial respiratory chain, SCO2-transfected LNT-229 p53sh cells were exposed to moderate hypoxia (1% $O_2$) in the presence or absence of rotenone, an inhibitor of complex I of the respiratory chain. Rotenone profoundly repressed oxygen consumption in LNT-229 p53sh cells transfected with either control vector or SCO2 (Fig. 5A). Hypoxia-induced cell death was unaffected by rotenone in LNT-229 p53sh cells transfected with control vector. The repression of cell death observed in SCO2-transfected cells was partly reverted by rotenone (Fig. 5B), indicating the requirement of a functional respiratory chain for SCO2´s function. Similar results were obtained in LNT-229 p53ts cells and in parental cells (data not shown). Supplementation of glucose rescued glioma cells irrespective of p53 status from hypoxic cell death suggesting that energy depletion is causal for the loss of cellular viability (Fig. 5 C). In contrast, methyl-pyruvate, a mitochondrial substrate, suppressed hypoxia-induced cell death only in p53 wild-type cells (Fig. 5 D). Cellular ATP concentrations in p53-depleted cells were decreased in hypoxia compared to p53 wild-type or SCO2 restored cells (Suppl. Fig. 1). However, no SCO2-dependent changes in the phosphorylation level of the energy-sensing kinase p-AMPKα or of its downstream targets actetyl coA carboxylase (ACC) or 4-EBP1 occurred (Suppl. Fig. 2). As SCO2 still had protective effects even in the presence of the complex I inhibitor rotenone, additional mechanisms independent of SCO2's function in oxidative phosphorylation might exist. Because SCO2 has recently been shown to suppress ROS levels (Sung, 2010), we investigated whether ROS levels would be modulated by SCO2 under starvation conditions, too. Indeed, intracellular ROS concentrations were reduced by ectopic expression of SCO2 (Suppl. Fig. 3), supporting a role for SCO2 in decreasing ROS levels.

**Suppression of SCO2 does not impact on clonogenicity *in vitro***

To analyze if p53 or SCO2 modulate clonogenicity of colon cancer cells in vitro under physiological conditions (3% $O_2$ and 5.5 mM glucose), anchorage independent growth was assessed by soft agar colony formation assays. To study in detail the role of SCO2, HCT116 $p53^{+/+}$ cells were stably transfected with a short-hairpin RNA targeting SCO2 (SCO2 sh) via a lentiviral system, resulting in about 75% knock-down in RT-PCR analysis (data not shown). Knock-out of p53 significantly impaired the ability of anchorage independent growth, whereas no such effect was observable in the SCO2-suppressed cells (Fig. 6). These results indicate that SCO2 does not impair clonogenic capacity *per se* in the presence of sufficient nutrients.

**Depletion of SCO2 promotes necrosis formation.**

As our *in vitro* models might not appropriately reflect the metabolic environment of tumor cells *in vivo*, we further analyzed the growth of HCT116 $p53^{+/+}$, $p53^{-/-}$, $p53^{+/+}$ scrambled sh and -SCO2 sh cells in a xenograft nude mouse model. No significant difference of the tumor growth doubling times could be observed between $p53^{+/+}$ (4.55 ± 0.66 d) and $p53^{-/-}$ (4.26 ± 0.99 d) tumors (Fig. 7A), but there was a tendency to produce larger tumors in HCT $p53^{-/-}$ cells at the end of the experiment (volumes at day 25 of HCT $p53^{+/+}$ vs. HCT $p53^{-/-}$ tumors: 1321 ± 642 $mm^3$ vs. 1718 ± 833 $mm^3$, mean ± SD, $p>0.05$) in accordance with previous reports using similar cell numbers (Bhonde *et al.*, 2006; Buzzai *et al.*, 2007; Yoon *et al.*, 2009). Similarly, SCO2-suppressed tumors did not have significant altered doubling times compared to control tumors (6.3 ± 1.52 d vs. 5.3 ± 0.5 d), but the tumor volumes at the end of the experiment were slightly larger in SCO2-depleted tumors (volumes at day 31 of scr sh vs. SCO2 sh tumors: 1293 ± 350 $mm^3$ vs. 1815 ± 641 $mm^3$, mean ± SD, $p<0.05$, Fig. 7B). Because faster glucose depletion of SCO2-suppressed tumor cells *in vitro* (Fig. 2 and data not shown) might be expected to result in altered necrosis formation *in vivo*, the extent of necrosis in comparison to the total tumor area was determined. Whereas in control tumors, necrosis

had a patchy distribution pattern with islets of viable tumors within necrotic areas, SCO2-suppressed tumors indeed revealed extensive central necrosis without viable tumors cells. Further, when the proportion of necrosis compared to the total tumor area was quantitatively analyzed, SCO2-suppressed tumors were characterized by significantly more necrosis compared to control tumors (Fig. 7C).

**Discussion**

p53 has recently been demonstrated to possess important functions in the control of cellular metabolism that may govern adaptive responses of tumor cells to their microenvironment. First, by mediating AMP kinase-induced growth arrest, p53 indirectly limits glucose consumption under starvation conditions (Jones *et al.*, 2005). Second, the p53 target TIGAR inhibits glycolysis and increases metabolism in the pentose phosphate shunt (Bensaad *et al.*, 2006). Third, SCO2 has been shown to be a p53 target gene (Matoba *et al.*, 2006) important for the assembly of the COX subunit within the inner mitochondrial membrane, thereby promoting cellular respiration (Leary *et al.*, 2004; Leary *et al.*, 2009). In summary, p53 serves as a key metabolic regulator limiting glucose consumption and increasing energy efficacy.

In primary glioblastoma, despite its cell cycle-regulating and proapoptotic functions, p53 is retained in its wild-type state in almost all cases (Fults *et al.*, 1992; Ohgaki and Kleihues, 2007; Van Meir *et al.*, 1994). In contrast, the receptor tyrosine kinase pathway is typically activated in these tumors leading to increased cellular metabolic demand and glucose consumption. Therefore, we hypothesized that the retention of wild-type p53 serves to adjust glucose metabolism in order to enable survival under the starvation conditions characteristic of the tumor microenvironment.

We here report that, indeed, under hypoxia, inactivation of p53 increased glucose consumption and cell death. Expression of SCO2 was downregulated and oxygen consumption was suppressed by antagonism of p53 (Fig. 3) consistent with the observation

that SCO2 is regulated by p53 (Matoba et al., 2006). Transfection of SCO2 into p53 deficient cells reestablished the metabolic phenotype of p53 wild-type cells enabling cellular respiration and enhancing cellular survival (Fig. 4). Most likely, this is due to the better efficiency of mitochondrial respiration compared to glycolysis, with 36 mol of ATP generated by oxidative phosphorylation versus 2 mol generated in the glycolytic pathway. Excess glucose rescued hypoxia-induced cell death in glycolytic (p53 mutant) as well as oxidative (p53 wild-type or SCO2 transfected) cells (Fig. 5 C), supporting the assumption that energy depletion is causal for the loss of viability under hypoxia and that glucose can be metabolized irrespective of p53 status under these conditions. Supplementation with methyl-pyruvate rescued survival only in p53 wild-type cells, but not in p53 mutant cells (Fig. 5 D). This is indicative of a functional p53-dependent respiration and of a selective aptitude of p53 wild-type cells compared to p53 mutant cells to metabolize mitochondrial substrates. p53 wild-type and SCO2 restored cells indeed maintained higher ATP levels under stress conditions than p53 mutant cells (Suppl. Fig. 1). However, although an increase in the phosphorylation level of the energy-sensing kinase AMPKα and subsequent modulation of its downstream targets acetyl coA carboxylase (ACC) or 4-EBP1 were detectable under hypoxic conditions, these adaptive responses were independent of the SCO2 expression (suppl. Fig. 2). Therefore, during hypoxia and glucose depletion, AMPK is activated irrespective of SCO2 status. We hypothesize that AMPK then triggers a starvation response that is more efficient in p53 wild-type or SCO2 expressing cells because these are able to utilize energy-effective oxidative phosphorylation while p53 mutant or SCO2 deficient cells deplete their glucose stores more rapidly (see also Fig. 2). The ensuing profound ATP depletion resulting in cell demise is possibly too rapid to regulate, in turn, phospho-AMPK to higher levels in p53 mutant / SCO2 deficient cells.

Since SCO2 expression did not decrease cell death under profound hypoxia, SCO2 seems to specifically act as a hypoxia-protective protein in the presence of at least traces of oxygen.

Furthermore, the assumption that SCO2-mediated protection from hypoxia-induced cell death is related to its function at the mitochondrial respiratory chain and requires an intact oxidative phosphorylation is supported by the observation that the protective effect of SCO2 is attenuated by inhibition of oxidative phosphorylation with the complex I inhibitor rotenone (Fig. 5). Since the protective effect of SCO2 under hypoxia was only partially reverted by inhibition of the respiratory chain, we wondered if SCO2 might protect cells from stress conditions by additional mechanisms. As shown in Suppl. Fig. 3, SCO2 suppressed intracellular ROS concentrations in LNT-229 and T98G cells both at normoxia and hypoxia. Our results are in agreement with findings in colon carcinoma cells and fibroblasts (Sung, 2010) and suggest that the suppression of ROS could be an additional cytoprotective mechanism of SCO2. How SCO2 suppresses ROS levels in glioma cells is unclear but might involve reduced intracellular oxygen concentrations (Sung, 2010).

Considering the complex tumor microenvironment, SCO2 might support the viability of tumor cells by increasing energy efficacy in the presence of at least threshold levels of oxygen and thereby reduce glucose consumption. The less efficient glucose metabolism of SCO2-lacking tumor cells could therefore be expected to result in increased necrosis formation. Indeed, the amount of necrosis was significantly increased in SCO2-depleted tumors compared to control tumors (Fig. 7). Similarly, inhibition of monocarboxylate transporter 1 (MCT-1) has been shown to result in reduced oxidative phosphorylation and increased glucose consumption of tumor cells near blood vessels, leading to glucose depletion of tumor cells in the tumor core and subsequent extensive necrosis in these areas (Sonveaux *et al.*, 2008).

In summary, this is the first report of SCO2 as a p53-dependent regulator of hypoxia sensitivity in glioma cells. Our results may have important implications for the understanding of the genetic basis of primary glioblastoma and may guide novel approaches towards tumor-

selective therapies. Given the fundamental importance of p53 as a tumor-suppressor (Efeyan and Serrano, 2007; Kim *et al.*, 2009) and the common loss of p53 in secondary glioblastomas (Ehrmann *et al.*, 1995; Van Meir *et al.*, 1994), the absence of p53 mutations in primary glioblastomas and the mutually exclusive occurrence of EGFR amplification and p53 mutations have long puzzled researchers. The increasingly evident role of hypoxic stress as a key selective pressure for solid tumors (Anderson *et al.*, 2006) has promoted research that aims to elucidate how cancer cells detect and process starvation signals and which pathways mediate adaptive responses that maintain viability (Jiang and Liu, 2008). Better understanding of these mechanisms may be exploited in several ways: (i) to establish molecular profiles that predict the susceptibility of cancer cells towards hypoxia. This will be of considerable importance, since common clinical interventions modulate tumor hypoxia. Examples include antiangiogenic therapies that increase tumor hypoxia, blood transfusions, erythropoietin application or oxygen delivery that decrease tumor hypoxia (Jansen *et al.*, 2004; Puduvalli and Sawaya, 2000; Rieger *et al.*, 2009) (ii) Interference with the adaptive responses towards starvation conditions by targeting key regulators of energy metabolism hold great promise as selective anti-tumor strategies. Specifically, SCO2 is a candidate molecule for this, because its suppression seems to be toxic only under hypoxic conditions. Co-therapy with strategies that target the availability or transport of glucose (Chan *et al.*) or the activity of glycolytic enzymes may further enhance the impact of suppression of SCO2 on tumor cells, because it will make them more dependent on cellular respiration. Strategies simultaneously targeting oxic and non-oxic metabolic pathways may therefore become an important component of novel antitumor therapies.

## Materials and methods

### Cell lines

LNT-229 carrying wild-type p53 activity (Lausanne, Switzerland) (Wischhusen et al., 2003) and LN-18 (endogenously mutant p53) cells were kindly provided from N. de Tribolet. T98G (endogenously mutant p53) were obtained from ATCC (Rockville, MD). LNT-229 cells expressing a short-hairpin construct for the gene suppression of p53 (LNT-229 p53sh) and puromycin-resistant control cells transfected with the empty vector (LNT-229 puro) (Wischhusen *et al.*, 2003) and LNT-229 cells stably expressing the temperature-sensitive murine $p53^{V135A}$ possessing dominant-negative properties at 38.5°C (LNT-229 p53ts) and hygromycin-resistant control cells transfected with the empty vector (LNT-229 hygro) have been described (Naumann *et al.*, 1998). Human HCT116 colon carcinoma cells carrying wild-type p53 ($p53^{+/+}$) and HCT116 cells with a targeted deletion of p53 ($p53^{-/-}$) were provided by B. Vogelstein (Bunz et al., 1998). SCO2 short hairpin RNA in pLKO.1 was stably transduced into HCT116 $p53^{+/+}$ cells (HCT116 $p53^{+/+}$-SCO2 sh) and selected with puromycin for 10 days. A scrambled shRNA in pLKO.1 was used as control (HCT116 $p53^{+/+}$-scrambled sh). Gene expression was confirmed by RT-PCR. The glioma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (PAA, Coelbe, Germany) containing 10% fetal calf serum (FCS), 2 mM glutamine, 100 IU/mL penicillin and 100 mg/mL streptomycin. LNT-229 hygro, LNT-229 p53ts cells and transfectants derived from those cells were cultivated at 38.5°C. In experiments requiring defined glucose concentrations, glucose as required was added to DMEM glucose free medium (PAA). Cells were seeded at a density of $5.7x10^4$ cells/$cm^2$ if not otherwise specified.

**Constructs**

Gene suppression of p53 was accomplished with the pSUPERpurop53shRNA vector (Wischhusen *et al.*, 2003). The hygromycin control vector and the $p53^{V135A}$ construct were a gift from M. Clarke. The p53-luc reporter gene vector PathDetect p53 was purchased from Stratagene (Cedar Creek, TX), pRL-CMV Renilla vector was from Promega (Mannheim,

Germany). pcDNA3.1-SCO2 was generously provided by P. Hwang (Matoba *et al.*, 2006). The control pcDNA3.1 vector was purchased from Invitrogen (Karlsruhe, Germany). Stable and transient transfections of plasmids were achieved using Metafectene pro (Biontex, Martinsried/Planegg, Germany) for LNT-229 or T98G cells. For stable knock down of human SCO2 expression, five different SCO2 shRNA (Sigma Aldrich, Taufkirchen, Germany) were each stably transduced into HCT116 $p53^{+/+}$ cells via a pLKO.1-based lentiviral system. The envelope plasmid 8454 pCMV-VSV-G and packaging plasmid 8455 pCMV-dR8.2 dvpr were purchased from Addgene (Cambridge, MA, USA). Protocol was adapted according to a previous report (Stewart *et al.*, 2003). Experiments were carried out with the most effective SCO2 shRNA sequence (Sigma aldrich, TRCN0000236560, 5′ CCGGAGTTACCGCGTGTACTACAATCTCGAGATTGTAGTACACGCGGTAACTTTT TTG-3′). A scrambled shRNA in pLKO.1 was used as control. For transient gene suppression of human SCO2, a predesigned small interfering RNA (siRNA) was employed (Sigma Aldrich, Taufkirchen, Germany). The following sequence targeting the coding region was used: 756-774: 5′-CAGUUACCGCGUGUACUAC [dT] [dT])-3′. A scrambled siRNA was used as control (Allstars negative siRNA, Qiagen, Hilden, Germany). Transient transfections with siRNA were achieved by HiPerfect (Qiagen) (3 µl Hiperfect : 20 nM siRNA per well in 24-well plates, seeded at 100.000 cells per well).

**Induction of hypoxia**

Profound hypoxia (0.1% $O_2$) was induced by incubating cells in Gas Pak pouches for anaerobic culture (Becton-Dickinson, Heidelberg, Germany) (Steinbach *et al.*, 2003). Moderate hypoxia (1% $O_2$) was induced in a Labotect incubator (Goettingen, Germany). Briefly, cells were seeded and allowed to attach in medium containing 10% FCS for 24 h. The medium was removed and the cells were incubated in serum-free DMEM adjusted to 2 mM

glucose under normoxia (21% $O_2$), moderate (1% $O_2$) or profound hypoxia (0.1% $O_2$) for the indicated periods of time.

**Immunoblot analysis**

Cells were seeded in 6 well plates and exposed to serum-free medium containing 2 mM glucose in the presence of 21%, 1% or 0.1% $O_2$ for 20 h as indicated. Thereafter, cells were washed with cold phosphate-buffered saline (PBS) and lysed in lysis buffer (50 mM Tris-HCl pH 8, 120 mM NaCl, 5 mM EDTA, 0.5% NP-40) containing protease inhibitors (Roche, Mannheim, Germany). Cellular lysates were prepared as described (Steinbach *et al.*, 2003) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Membranes were probed with antibodies to human p53 (sc-263, Santa Cruz, San Diego, CA), murine p53 (p53-Ab-4, Merck, Darmstadt, Germany), GAPDH (MAB374, Chemicon, Nuernberg, Germany), AMPKα (2531, Cell Signaling, Frankfurt am Main), p-AMPKα (Cell Signaling 2531, Germany), ACC (3662, Cell Signaling), p-ACC (3661, Cell Signaling), actin (SC1616, Santa Cruz) or p-4-EBP1 (9459, Cell Signaling). Secondary antibodies were purchased from Santa Cruz. The chemiluminescence solution used for detection was composed of 1 ml solution A (200 ml 0.1 M Tris-HCl pH 8.6, 50 mg Luminol), 100 µl solution B (11 mg p-hydroxycurmarinacid, 10 ml DMSO) and 0.3 µl $H_2O_2$ (30%).

**Luciferase assay**

Cells were seeded at 10000 cells per well into 96 well plates, cotransfected using Metafectene pro (Biontex) with the p53-luc reporter gene vector and pRL-CMV (Renilla) vector (Promega, Mannheim, Germany) at a ratio of 7.5 : 1, and exposed to 2 mM glucose and oxygen concentrations for 16 h. Experiments were conducted in triplicates. Activities of luciferase and firefly were determined using a luminometer (Mithras). Background was subtracted from

all values and the counts obtained from the measurement of firefly luciferase were normalized to Renilla luciferase (Dyer *et al.*, 2000; Wischhusen *et al.*, 2004).

**Cell death analysis**

Cells were seeded in 24 well plates at 50.000/$cm^2$. After 24 h, medium was removed and cells were incubated in DMEM containing 2 mM glucose in the presence of 21%, 1% or 0.1% $O_2$ for 20 h. Thereafter, adherent and non-adherent cells were collected, washed in PBS, stained with 1 µg/ml PI, and analyzed by flow cytometry in a BD Canto II. PI-positive cells were regarded as dead cells, and the percentage of dead cells in relation to all analyzed cells was determined. Experiments were performed in triplicates and are presented as mean ± standard deviation (SD). Selected results were replicated by LDH assay (Roche, Mannheim, Germany) according to manufacturer's instructions.

**ROS analysis**

Cells were seeded in 24 well plates. After 24 h, medium was removed, and cells were incubated in DMEM containing 5 mM glucose in the presence of 21% or 1% $O_2$ for 20 h. Cells were then washed twice with PBS, incubated 20 min, 37°C with 10 µM $H_2$DCFDA (Invitrogen, Karlsruhe, Germany), washed with PBS and collected for flow cytometry analysis. DCF signal was analyzed by BD Canto II. Experiments were performed in triplicates and are presented as mean ± standard deviation (SD).

**Measurement of glucose and lactate**

Cell-free supernatant was collected and glucose and lactate concentrations were measured using the biochemistry analyzer Hitachi 917.

**RNA extraction and quantitative reverse transcription-PCR (qRT-PCR) analysis.**

Total RNA was extracted using Trizol and RNAeasy Kit (Invitrogen, Karlsruhe, Germany). First strand cDNA was synthesized using the Vilo cDNA synthesis kit (Invitrogen) for 10 min at 25°C and 2 h at 42°C. Subsequently, the enzyme was inactivated at 85°C for 10 min. To determine changes in gene expression, qRT-PCR was performed in the IQ5 real-time PCR detection system (Biorad, Muenchen, Germany) using Absolute Blue Q-PCR Mastermix with SybrGreen+Fluorescein (Thermo Fisher Scientific, Hamburg, Germany) and the following primer pairs: SCO2 fw 5′-CTTCACTCACTGCCCTGACA-3′, SCO2 bw 5′-TGAGCAGGTAGATGGCAATG-3′, 18S fw 5′-CGGCTACCACATCCAAGGAA-3′, 18S bw 5′-GCTGGAATTACCGCGGCT-3′. Cycle threshold (Ct) values were normalized for amplification of the 18S ribosomal RNA and the data were analyzed using the Vandesompele method (28).

**Oxygen consumption**

250.000 cells were seeded in 3 cm diameter glass dishes. After 24 h, cells were treated with medium preincubated at the required temperature (37°C or 38.5°C), the lids were sealed using rubber gaskets and the dishes were tightly closed by two cramps. Oxygen concentration in the medium was measured using the ABL-80 FLEX Blood Gas Analyzer (Radiometer, Willich, Germany) before and after incubation for 48 h. Oxygen consumption was normalized for protein concentration.

**ATP assay**

Immediately after hypoxic incubation, the plates were placed on ice and the cells were pelleted by centrifugation and lysed in ATP releasing reagent (Sigma). The ATP concentration was determined by luciferase assay with the CLS II kit (Boehringer, Mannheim, Germany) (Steinbach et al., 2003).

**Anchorage independent growth (Soft agar colony assay)**

Cells were suspended in 0.35% agar medium (DMEM containing 5.5 mM glucose, 10% FCS, 2 mM GlutaMAX™-I Supplement (Invitrogen)) at a concentration of $5 \times 10^3$ cells per 6-cm dish, and then plated on a 0.5% agar base layer (also containing 5.5 mM glucose, 10% FCS and 2 mM GlutaMAX™-I Supplement). GlutaMAX™ contains L-alanyl-L-glutamine, a stabilized form of L-glutamine, preventing degradation and ammonia build-up during long-term cultures. Cells were then incubated in a humidified atmosphere (5% $CO_2$) at 37°C. 24 h after seeding, 2 ml of medium were added on the top agar and cells were incubated at 3% $O_2$ for at least 4 weeks. Colonies were stained by 0.0025% crystal violet for 4 h and counted thereafter.

***In vivo* assays**

4 x $10^6$ HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells and 2 x $10^6$ $p53^{+/+}$ scrambled sh or -SCO2 sh cells were injected subcutaneously (s. c.) in both lower back sides of 6-week-old female athymic nude mice (Foxn1nu, Harlan, Indianapolis, IN). 10 mice (corresponding to 20 tumors) were used for HCT116 $p53^{+/+}$ /$p53^{-/-}$ cells, 6 mice were used (12 tumors) for $p53^{+/+}$ scrambled sh / SCO2 sh cells. A smaller amount of cells in the scrambled/SCO2 sh model were used to observe tumors for a longer period of time. The size of the transplanted tumors was measured three times per week by means of a caliper. Tumor volume was calculated by use of the formula $\pi/6*\text{length}*\text{width}^2$ (Kopper and Steel, 1975). Animals were sacrificed before the average of tumor volumes reached 1.500 $mm^3$. Experiments were conducted according to the guidelines for ethical use of animals of the Helsinki declaration under an approved protocol. The doubling times of the subcutaneous tumors was calculated by dividing the natural logarithm of 2 by the exponent of an exponential growth curve calculated for each tumor. Thereafter, doubling times between the groups were compared by t-test.

**Necrosis fraction determination**

Shortly after surgical removal, tumors were fixed and embedded in 4% paraffin. Blocks were cut with a microtome (3 µm thickness), placed on Super-Frost Plus slides (Microm International, Walldorf, Germany) and stained with haematoxylin and eosin (H&E). Slices were scanned and the extent of necrosis in relation to total tumor area was determined using ImageJ software (Research Services Branch, National Institute of Mental Health, Bethesda). To analyze tumors of comparable size, tumors of less than 500 $mm^3$ were excluded from the analysis.

**Chemicals**

All chemicals not otherwise specified below were from Sigma Aldrich (Deisenhofen, Germany), Invitrogen (Karlsruhe, Germany) or Roth (Karlsruhe, Germany).

**Statistics**

Values were compared by two-tailed student´s t-test (Excel, Microsoft, Seattle, WA), and p-values of $p < 0.05$ and $p < 0.01$ were considered significant and highly significant.

**Conflict of interest**

The authors declare no conflict of interest.

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**Figure Legends**

**Fig. 1: Antagonizing p53 enhances hypoxia-induced cell death.** Three different model systems for the suppression of p53 function were employed. LNT-229 p53sh cells or puro control cells (upper panel), LNT-229 p53ts cells carrying the temperature-sensitive dominant-negative mutant $p53^{V135A}$ or hygro control cells (middle panel), and the HCT116 system with the $p53^{-/-}$ subline or the $p53^{+/+}$ parental cells (lower panel). (A) Tumor cells were incubated at 21%, 1% or 0.1% $O_2$, and the expression of endogenous human p53 (p53sc263-antibody) or murine p53 (Ab4-antibody) for the detection of the murine p53ts transgene were analyzed by Western blot. GAPDH expression was employed as loading control. (B) p53 transactivation activity under the denoted conditions was determined by a luciferase assay (mean ± SD). (C) Cells were treated as in (A), and cell death was analyzed by PI-FACS (left panel). Scatter plots are of representative experiments showing forward scatter vs. PI uptake. Cells above the horizontal gates shown in red were defined as dead cells (right panel). Relative percentages of dead cells under these conditions as determined in triplicates are shown (mean ± SD). * $p< 0.05$, ** $p<0.01$.

**Fig. 2: p53 is a regulator of glucose consumption in tumor cells.** LNT-229 puro and LNT-229 p53 sh cells (A-B) or HCT116 $p53^{+/+}$/$p53^{-/-}$ cells (C-D) were treated as denoted for the indicated periods of time, and glucose consumption (A, C) and lactate production (B, D) were assessed. A representative experiment of 3 independent experiments is shown.

**Fig. 3: Inhibiting p53 function represses cellular respiration and expression of SCO2.** LNT-229 puro/p53sh (left panel), LNT-229 hygro/p53ts (middle panel) or HCT116 $p53^{+/+}$/$p53^{-/-}$ cells (right panel) were analyzed for (A) SCO2 expression by quantitative RT-PCR (error bars represent standard deviation measured in three samples and standardized to

18S, * p< 0.05, ** p<0.01) and (B) oxygen consumption. For B, a representative experiment out of 3 independent experiments with similar results is shown.

**Fig. 4: SCO2 is a p53 effector that maintains respiration and reduces sensitivity towards hypoxic induced cell death.** LNT-229, T98G (p53 mutant) or LNT-229 puro/p53sh cells were stably transfected with pcDNA3-SCO2 (SCO2) or a control plasmid (neo). Further, LNT-229 cells were transiently transfected with a siRNA targeting SCO2 (SCO2si) or a scrambled siRNA (scr si). In these cell lines, (A) expression of SCO2 (error bars represent standard deviation measured in three samples and standardized to 18S, * p< 0.05, ** p<0.01), (B) oxygen consumption and (C) hypoxia-induced cell death (mean ± SD) were analyzed. * p< 0.05, ** p<0.01. For B, a representative experiment out of 3 independent experiments with similar results is shown.

**Fig. 5: A functional respiratory chain is required for the SCO2-mediated protection.** (A) LNT-229 p53sh cells ectopically expressing SCO2 (SCO2) or empty pcdna3 vector-transfected p53sh cells (neo) were exposed to rotenone (100 nM) or vehicle for 24 h, and oxygen consumption was determined (A). Additionally, cell death induced by moderate hypoxia (1%) in the absence or presence of rotenone was analyzed in these cells (mean ± SD). * p< 0.05, ** p<0.01 (B). T98G cells ectopically expressing SCO2 (SCO2) or empty pcdna3 vector-transfected T98G cells (neo) and LNT-229 hygro/p53ts cells stably transfected with pcDNA3-SCO2 (SCO2) or a control plasmid (neo) were exposed to serum-free media containing 2 or 25 mM glucose under moderate hypoxia (1% O2) for 24 h, and cell death was assayed by PI-FACS (mean ± SD), * p< 0.05, ** p<0.01 (C). LNT-229 hygro and LNT-229 p53ts cells were exposed to serum-free media containing 2 mM glucose with or without 10 mM methyl-pyruvate under moderate hypoxia (1% O2) for 24 h, and cell death was assayed

by PI-FACS (mean ± SD). * p< 0.05, ** p<0.01 (D). Here, a representative experiment out of 3 independent experiments with similar results is shown.

**Fig.6: Suppression of SCO2 does not impair clonogenicity *in vitro***

HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells (A) and HCT116 $p53^{+/+}$ scrambled sh/SCO2 sh cells (B) were seeded into a 3D agar matrix on top of a base agar layer in presence of 5.5 mM glucose and 3% $O_2$. After 4-6 weeks of incubation, colonies were stained by 0.0025% crystal violet and the number of colonies formed in each well was counted. The bars represent the average ± SD of three wells counted for each group. The results were confirmed in at least 3 independent experiments ** p < 0.001.

**Fig. 7: Depletion of SCO2 promotes necrosis formation.**

(A) HCT116 $p53^{+/+}$ and $p53^{-/-}$cells, and (B) HCT116 $p53^{+/+}$ scrambled sh/SCO2 sh cells were injected s.c. in the lower back side of 6-week-old female athymic nude foxn1 nu mice. The size of the transplanted tumors was measured three times per week with a caliper. Data are mean ± SEM, n.s.: no significant difference in doubling times. (C) After surgical removal, HCT116 $p53^{+/+}$ scrambled sh/SCO2 sh tumors were fixed and stained with H&E. (upper panel). Necrosis fraction of tumors was determined and compared by t-test (n=7 for scrambled sh and n=10 for SCO2 sh tumors, ** p < 0.001). (lower panel). Representative slices of scrambled sh and SCO2 sh tumors are shown.

**Fig. 8: Schematic drawing showing the regulation of hypoxia-induced cell death through p53-dependent expression of SCO2.** (A) In the presence of wild-type p53, starvation signals induce p53 via the AMPK sensor system. P53 suppresses glycolysis and induces SCO2, which promotes oxidative phosphorylation in the mitochondria. Under the conditions of the tumor microenvironment characterized by hypoxia and low glucose availability, increased

efficacy of ATP production in the mitochondria serves to maintain energy homeostasis and viability. Furthermore, expression of SCO2 limits ROS levels. (B) Inactivation of p53 or suppression of SCO2 deregulates glycolysis and impairs mitochondrial respiration leading to increased glucose consumption, while the amount of ATP generated per Mol glucose is decreased. Under starvation conditions, these metabolic alterations induce rapid energy depletion and subsequent cell death. Reduced expression of SCO2 results in increased intracellular ROS levels which may further contribute to cell death.

**Supplementary Figures:**

**Suppl. Fig. 1: SCO2 expression leads to higher ATP levels under moderate hypoxia.** LNT-229 hygro or p53ts cells stably transfected with pcDNA3-SCO2 (SCO2) or a control plasmid (neo) were exposed to serum-free media containing 2 mM glucose under moderate hypoxia (1% O2) for 16 h and ATP concentrations were measured as described (mean ± SD). * $p< 0.05$, ** $p<0.01$. A representative experiment out of 3 independent experiments with similar results is shown.

**Suppl. Fig. 2**: **SCO2 expression has no impact on phosphorylation of AMPKα or ACC under moderate hypoxia.** T98G cells ectopically expressing SCO2 (SCO2) or empty pcdna3 vector-transfected T98G cells (neo) were exposed to serum-free media containing 2 mM glucose under moderate hypoxia, and expression of ACC, phosphorylated ACC (p-ACC), AMPKα, phosphorylated AMPKα (p-AMPKα) and phosphorylated 4EBP1 (p-4EBP1) were analyzed by Western blot. The glycolysis inhibitor 2-deoxyglucose (2-DG) (5 mM) was employed as a positive control, and actin expression was used as loading control. A representative experiment out of 2 independent experiments with similar results is shown.

**Suppl. Fig. 3: SCO2 suppresses intracellular ROS concentrations in glioma cells.** LNT-229 and T98G cells ectopically expressing SCO2 (SCO2) or empty pcdna3 vector-transfected LNT-229 and T98G cells (neo) were exposed to serum-free media containing 5 mM glucose under normoxia or moderate hypoxia (1%) for 24 h, and ROS levels were determined. * $p < 0.05$. A representative experiment out of 3 independent experiments with similar results is shown.

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